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### Vitamin D biology and heart failure

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# Parental vitamin D deficiency during pregnancy is associated with increased blood pressure in offspring via Panx1 hypermethylation

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# Chapter

*Abstract*

Vitamin D deficiency is the most common nutritional deficiency worldwide. Maternal vitamin D deficiency is associated with increased susceptibility of hypertension in offspring but the reasons for this remain unknown. The aim of this study was to determine if parental vitamin D deficiency leads to altered DNA methylation in offspring that may relate to hypertension.

Male and female Sprague-Dawley rats were fed a standard or vitamin D depleted diet. After 10 weeks, non-sibling rats were mated the conceived pups received standard chow. We observed an increased systolic and diastolic blood pressure in the offspring from depleted parents (F1-depl). Genome-wide methylation analyses in offspring identified hypermethylation of the promoter region of the Pannexin-1(*Panx1*)-gene in F1-depl-rats. *Panx1* encodes a hemi channel known to be involved in endothelial-dependent relaxation, and we demonstrated that in F1-depl-rats the increase in blood pressure was associated with impaired endothelial relaxation of the large vessels, suggesting an underlying biological mechanism of increased blood pressure in children from parents with vitamin deficiency.

Parental vitamin D deficiency is associated with epigenetic changes and increased blood pressure levels in offspring.

## Introduction

Hypertension is a major risk factor in the development of cardiovascular (CV) disease and premature death worldwide. The prevalence of hypertension is on the rise due to the increasing ageing population, and it is estimated that in 2025 1.56 billion adults will be affected by this condition (1).

The underlying mechanisms of hypertension are complex and multifactorial, but amongst many others, heritable factors play a role (2,3). This may include epigenetic DNA modifications. Several groups have identified epigenetic markers that could contribute to increased susceptibility to develop hypertension (4-6) .

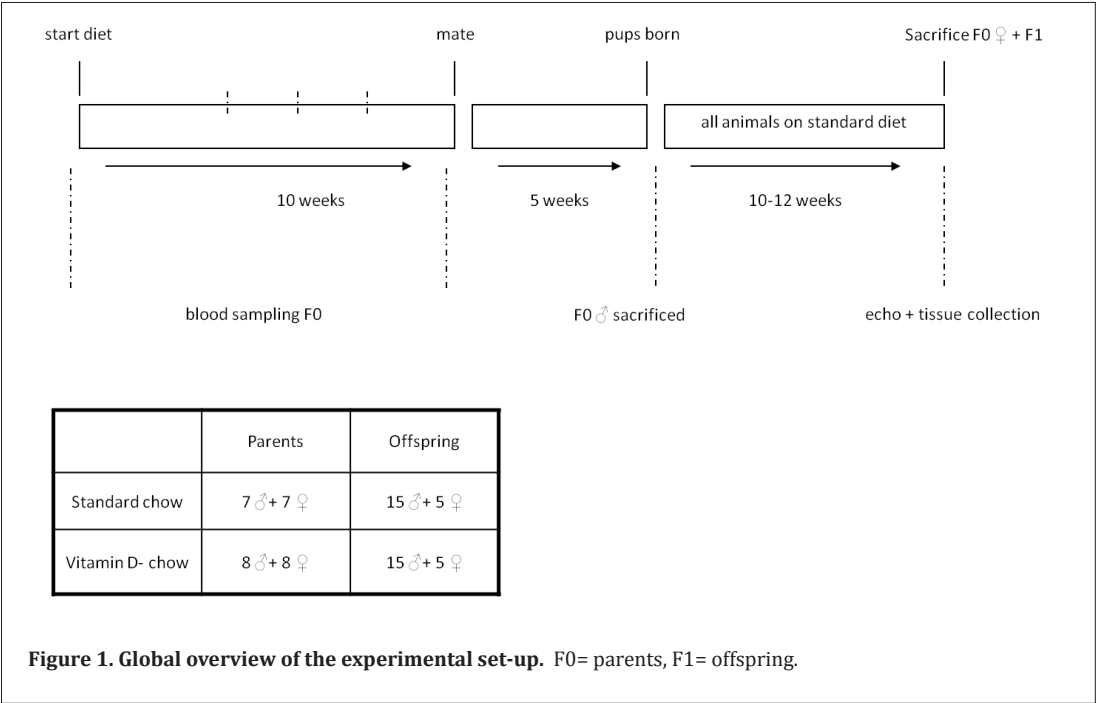
Recently, it became apparent that in humans maternal nutritional status during early pregnancy predictably affected DNA methylation in offspring (7). Previously, it had been demonstrated in mice that changes in maternal diet altered epigenetic development at genomic regions involved in DNA-methylation during embryonic phase (8-10). These data suggest that nutritional deficiencies may induce epigenetic changes that modify gene transcriptional activity and (indirectly) alter disease susceptibility (11). In light of this, one of the nutritional components that may be of particular interest is vitamin D. Due to our predominantly indoor lifestyles, vitamin D deficiency is in fact the most common nutritional deficiency worldwide (12,13). Interestingly, maternal vitamin D deficiency has been associated with the development of short and long term disorders in the offspring, including hypertension (13). However, the mechanism linking parental vitamin D deficiency to increased susceptibility for hypertension in the offspring remains to be elucidated. We used a rat model to study the consequences of parental vitamin D deficiency on blood pressure and epigenetic changes in offspring.

# Materials and Methods

*Animal Care and Experimental Design.* All animal studies were approved by the Animal Ethical Committee of the University of Groningen, The Netherlands, and conducted in accordance with existing guidelines for the care and use of laboratory animals.

We studied 12-week old single-caged Sprague-Dawley male and female rats (Harlan, the Netherlands), and supplied them with normal (n=7 males, 7 females) or vitamin D depleted diet (n= 8 males, 8 females) (Harlan [Teklad], diet code TD.87095) with 12:12 hr day-night cycles and *ad lib* access to tap water. After 10 weeks of dietary intervention, blood pressure was measured with a non-invasive method (tail-cuff method, rats were trained for 2 weeks beforehand). We collected blood (via venipuncture under anesthesia) from male and female animals to determine vitamin D status (25-hydroxyvitamin D (25(OH)D) and parathyroid hormone (PTH) levels).

*Experimental procedures.* After 10 weeks, a single female was mated with one non-sibling male from the same dietary group, and dietary treatment was continued. Following delivery, males were removed from the cage and sacrificed, while females and offspring were maintained group-caged. From this point on, all animals were supplied with a standard diet for the entire duration of subsequent experiments (Figure 1). F1 pups were nursed freely and weaned at 3 weeks onto standard chow, provided *ad libitum*.



After 2 weeks of training, final blood pressure measurement was performed in 10 6-week old F1-pups (10 male, 10 female). 10 other age-matched male pups per dietary arm received telemeter implantation (Data Sciences International, St. Paul, MN, USA). Telemeters were used to continuously monitor blood pressure with a sampling frequency of 10s every 10 minutes for 72 hours.

*Echocardiography and hemodynamic measurements.* Female rats and F1 pups were sacrificed 10-12 weeks after delivery. Approximately 1 week prior to sacrifice, echocardiography was performed using a transthoracic echocardiography with a 10 MHz transducer (Vivid 7, GE Healthcare, Diegem, Belgium) as previously described (14). Briefly, rats were anaesthetized (2.5% isoflurane) and body temperature was maintained by placing the rat on a heating pad. Short-axis view and M-mode tracings were used to measure cardiac hypertrophy and heart dimensions.

Prior to sacrifice, hemodynamic function was measured, using an indwelling pressure tip catheter (Millar Instruments, Houston, TX, USA), that was introduced in the right carotid artery and advanced into the LV as previously described (14). After measuring invasive hemodynamics, blood was drawn via cardiac puncture and hearts together with other organs were rapidly excised and weighed. Myocardial and kidney tissue were dissected transversally and snap frozen for molecular analyses. Thoracic aortas were collected for vascular studies, while abdominal aortas were collected and snap frozen for molecular analyses.

*Enzymeimmunoassay (EIA) of 25(OH)D.* Plasma 25(OH)D levels were analyzed using a commercial Enzyme immunoassay according to the manufacturer's protocol (25-Hydroxy Vitamin D EIA kit, AC-57F1, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany).

*Enzyme-linked immunosorbent assay (ELISA) of PTH.* Plasma PTH levels were analyzed using a commercial Enzyme Linked Immunosorbent Assay according to the manufacturer's protocol (Rat Intact PTH ELISA Kit, #60-2500, Immunotopics Inc., San Clemente, CA).

*Vascular studies with isolated aorta rings.* The thoracic descending aorta was excised and placed in a Krebs bicarbonate solution of the following composition (mmol·L<sup>-1</sup>): NaCl, 120.4; KCl, 5.9; CaCl<sub>2</sub>, 2.5; MgCl<sub>2</sub>, 1.2; NaH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 11.5; NaHCO<sub>3</sub>, 25.0; continuously aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. The vessel was cleaned of adhering fat tissue and rings of 2 mm in width were cut with a sharp razor blade, whilst ensuring not to touch the luminal surface. Rings were mounted between two stirrups in organ baths filled with

15 ml of Krebs solution. One stirrup was anchored inside the organ bath while the other was connected to a displacement transducer to determine isotonic changes, as previously described (15). Rings were then subjected to 14 mN and allowed to stabilize for 60 min before they were primed and checked for viability by evoking a contraction with 60 mmol/L KCl twice. Following washout and renewed stabilization, parallel rings were studied (in duplicate) either for contractile responses to KCl (10 to 80 mmol/L) or Phenylephrine (PE, 1 nmol/L to 10  $\mu$ mol/L), or endothelium-dependent relaxation. For the latter, rings were pre-constricted with 1  $\mu$ mol·L<sup>-1</sup> PE followed by determination of the dilatory response to the endothelium-dependent vasodilator acetylcholine (ACh: 10 nmol·L<sup>-1</sup> to 10  $\mu$ mol·L<sup>-1</sup>). Subsequently, a single high concentration of sodium nitroprusside (SNP, 10  $\mu$ mol/L) was administered to determine maximal endothelium-independent relaxation.

*Quantitative real-time PCR.* To measure mRNA gene expression levels, total RNA from left ventricle (LV) and kidney tissue was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). cDNA synthesis and quantitative real time PCR (RT-qPCR) were performed as previously described with use of 0.5  $\mu$ g total RNA (16). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of cDNA mixture. RNA expression data was normalized using the 36B4 as a reference. We assayed transcript abundance of genes listed in Table 1.

*Genomic DNA isolation, genome-wide methylation status and data analysis.* DNA was extracted from heart and kidney tissue, using the prepGEM Tissue 200 kit (ZyGem, New Zealand). The methylated DNA immunoprecipitation (MeDIP) was labeled with Cy5 fluorophore and the input genomic DNA was labeled with Cy3 fluorophore. The labeled DNA samples were combined and hybridized to Rat 3x720K Multiplex CpG Island Plus RefSeq Promoter Array (Nimblegen).

Genome-wide methylation status was determined using a multiplex Rat Nimblegen 3x720K CpG Island Plus RefSeq Promoter Array (Roche Nimblegen, Inc., Madison, USA). These arrays include 15,287 RefSeq gene promoters and 15,790 annotated CpG islands with 100 bp spacing through all tiled regions. Tiling of RefSeq promoters begins 3.9kb upstream, extends downstream 0.97kb and covers a total of 4.87kb of promoter per gene. The probe lengths were between 50–75 mer. Arrays were scanned using a MS200 scanner (Nimblegen). Data were extracted and exported to excel using NimbleScan. For MeDIP data the log<sup>2</sup>-ratios of fluorescence intensity obtained from the scanner were pre-processed by median-centering and quantile normalization as proposed by Pålmeke *et al.* (17) using the Bioconductor packages Ringo (18) and *limma* (19). To define differentially methylated regions, a combination of three separate algorithms was applied, compromising of two

publicly available methods BATMAN (20) and dmrFinder from the CHARM package (21) as well as a self-programmed R-script.

*Assessment of DNA methylation status by pyrosequencing.* According to the DNA methylation quantification-method of Tost and Gut, 2007, we analyzed the quantitative DNA methylation status of the Pannexin-1 (*Panx1*) gene by pyrosequencing of bisulfite-treated DNA (22). Briefly, 1 µg of DNA from heart or kidney tissue, respectively, was bisulfite-converted using the EZ DNA Methylation™ Kit (Zymo Research). Regions of interest selected for validation were amplified using 2µL of bisulfite-treated genomic DNA and 10 µM of forward and reverse primer; one of them being biotinylated (22). Information about PCR and sequencing primers and the sequence-to-analyze is summarized in table 1. Quantification of the DNA methylation status of the *Panx1* was facilitated on a PyromarkQ24 system (Qiagen) as described by Freitag *et al.* (23). Results were analyzed the results using the PyroMark CpG software (V.10.0.11.14, QIAGEN).

**Table 1** - Overview of primers used for rt-PCR and DNA methylation status quantification.

Primers used for RT q-PCR	
36B4 (forward)	GTTGCCTCAGTGCTCACTC
36B4 (reverse)	GCAGCCGCAAATGCAGATGG
Angiotensin 1 Receptor (forward)	CTGGTTCACTGGCTGTTGAC
Angiotensin 1 Receptor (reverse)	GGAAGATGGTGGAACCAAG
Atrial natriuretic peptide (forward)	ATGGGCTCCTTCTCCATCAC
Atrial natriuretic peptide (reverse)	TCTACCGGCATCTTCTCCTC
Pannexin-1 (forward)	AGACCAAGGGAGAGGACCA
Pannexin-1 (reverse)	GCTGCTCAGGTCCAAATCTT
Renin (forward)	GTTGCTCTGGACCTCTTGTA
Renin (reverse)	CACTGATCCTGGTCATGTCT
Vitamin-D Receptor (forward)	GAGATTGCCGATCACCAAG
Vitamin-D Receptor (reverse)	ACGCTGTACCTCCTCATCTG
Primers used for pyrosequence	
Pannexin-1 (forward)	GGTTAGTAGGAGATAGGGTGGGGTTA
Pannexin-1 (reverse, biotinylated)	ATCACACCCCTACTCAACTCCC
Pannexin-1 (sequencing)	TTTTAGGTAAGTTGTTGTTT
Sequence (CpG-island) to analyze of <i>Panx1</i>	
TTGYGYGTTT AAGGYGGTYG GAGTTTTGGT GTGYGYGYG TTGGGGATTY G	



*Statistical analyses.* Results were reported as means  $\pm$  SEM, unless reported otherwise. Statistical analysis between groups was performed by using the Student's-*t*-test if data was normally distributed or the Mann-Whitney U test if data was not normally distributed. Blood pressure-curves were analyzed using repeated measures ANOVA analysis. All *p* values were two-tailed and *p* values  $<0.05$  were considered statistically significant.

Aorta contractile responses to KCl and PE were given in  $\mu\text{m}$ . Vasodilator responses to ACh were expressed as a percentage of PE-induced pre-contraction before contraction-tension response curves were generated (Graphpad Prism 5, Graphpad Software, San Diego, CA). *N* values represent the number of investigated rats. Full contraction-tension response curves were compared using repeated measurements ANOVA. Analyses were performed using STATA version 11.0 software (STATACorp, Texas, USA).

For BATMAN analysis, the difference in methylation between sets of windows of two different groups was assessed by a Wilcoxon-Mann-Whitney test for every step. As for the other approaches (dmrFinder and self-programmed R-script) the combined use of several arrays per phenotype led to an increased possibility to detect differences while the impact of potential artefacts was reduced. Whilst searching for differentially methylated regions we selected only those regions that displayed significant differences for at least three consecutive sliding windows at a significance level of  $p=0.001$ .

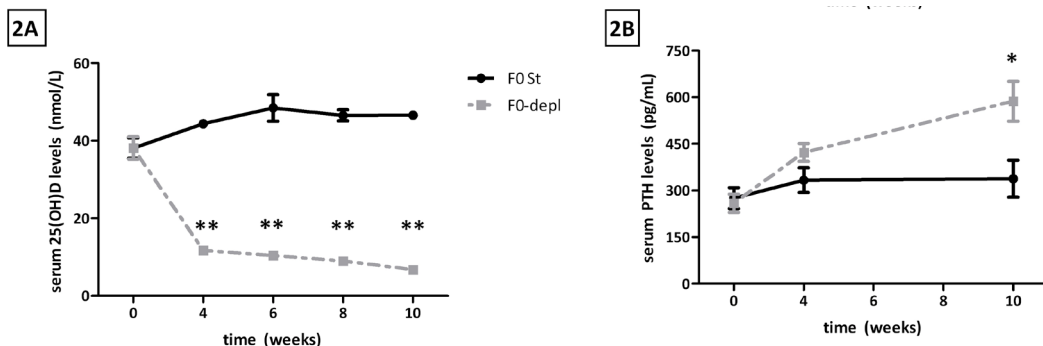
## Results

*Vitamin D deficient diet resulted in a pronounced and prolonged vitamin D deficiency in F0-rats.* F0-rats (parents) were fed with standard or vitamin D depleted diet for 10 weeks. We dietary effect on plasma 25(OH)D and PTH levels was verified before rats were mated (Figure 2). Baseline characteristics and echocardiography data of F1-rats are presented in table 2. Offspring from parents fed a standard diet (F1-st) were compared with offspring from parents fed on a vitamin-D depleted diet (F1-depl). We observed no differences in body weight (BW), total heart weight (HW) or left ventricular weight (LVW) (Table 2). Furthermore, we calculated HW/BW-ratio and LVW/BW-ratio as a relative measure of cardiac hypertrophy. Again, outcomes were not different between groups (Table 2). Echocardiography was performed to assess the effects of a vitamin D depleted diet during pregnancy on cardiac performance in the offspring. Although global cardiac function was similar in all rats, we observed a minimally impaired cardiac function in F1-depl-rats, as reflected by the increased LV internal diameter and decreased E-velocity, fractional shortening and ejection fraction (Table 2).

**Table 2** - Baseline characteristics and echocardiography data of F1-rats (mean + SEM)

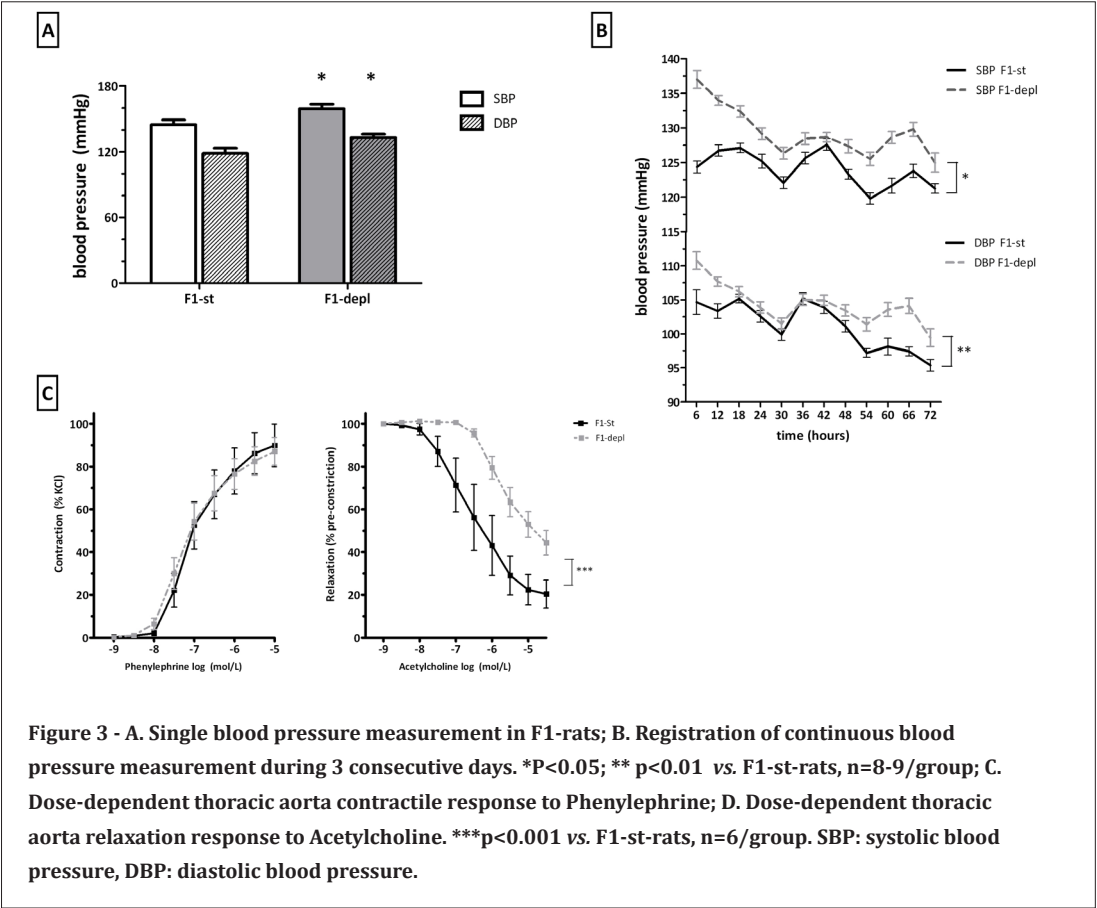
	F1-st (N=10)	F1-depl (N=10)
BW (g)	336 ± 8	325 ± 6
HW (mg)	1092 ± 17	1060 ± 17
LVW (mg)	825 ± 13	798 ± 13
HW/BW (g/mm)	3.3 ± 0.2	3.3 ± 0.2
LVW/BW (g/mm)	2.5 ± 0.2	2.5 ± 0.1
HR (bpm)	398 ± 7	397 ± 8
MV A (m/s)	0.68 ± 0.05	0.63 ± 0.05
MV E (m/s)	1.04 ± 0.02	0.89 ± 0.05*
MV E/A ratio	1.60 ± 0.11	1.50 ± 0.06
IVSs (mm)	2.78 ± 0.05	2.64 ± 0.05
LVIDs (mm)	2.69 ± 0.18	3.34 ± 0.10**
LVPWs (mm)	2.82 ± 0.07	2.79 ± 0.05
IVSd (mm)	1.62 ± 0.07	1.56 ± 0.03
LVIDd (mm)	5.86 ± 0.22	6.34 ± 0.09*
LVPWd (mm)	1.63 ± 0.05	1.56 ± 0.04
LVOT diam (mm)	2.97 ± 0.04	2.92 ± 0.06
EF(%)	88 ± 1.6	84 ± 0.7*
%FS	54 ± 2.2	48 ± 0.8 *
LV CO (ml/min)	147 ± 8	135 ± 8

Table 2 shows the baseline characteristics and echocardiographic data at sacrifice of the used rats. Data is expressed as mean ± SEM. st = standard chow, depl = vitamin D deficient chow, BW = body weight, HW= heart weight, LVW = left ventricular weight, TL = tibia length, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, HR = heart rate, bpm = beats per minute, A = late ventricular filling velocities, E= early ventricular filling velocities, E/A ratio= diastolic relaxation, IVSs = thickness of the interventricular septum in systole, LVIDs= left ventricular internal diameter in systole, LVPWs = thickness of the left ventricular posterior wall in systole, IVSd = thickness of the interventricular septum in diastole, LVIDd= left ventricular internal diameter in diastole, LVPWd = thickness of the left ventricular posterior wall in diastole, LVOT = left ventricular outflow tract, EF= ejection fraction, FS= fractional shortening, CO= cardiac output. \* P<0.05 F1-st vs. F1-depl, \*\* P< 0.01 F1-st vs. F1-depl.



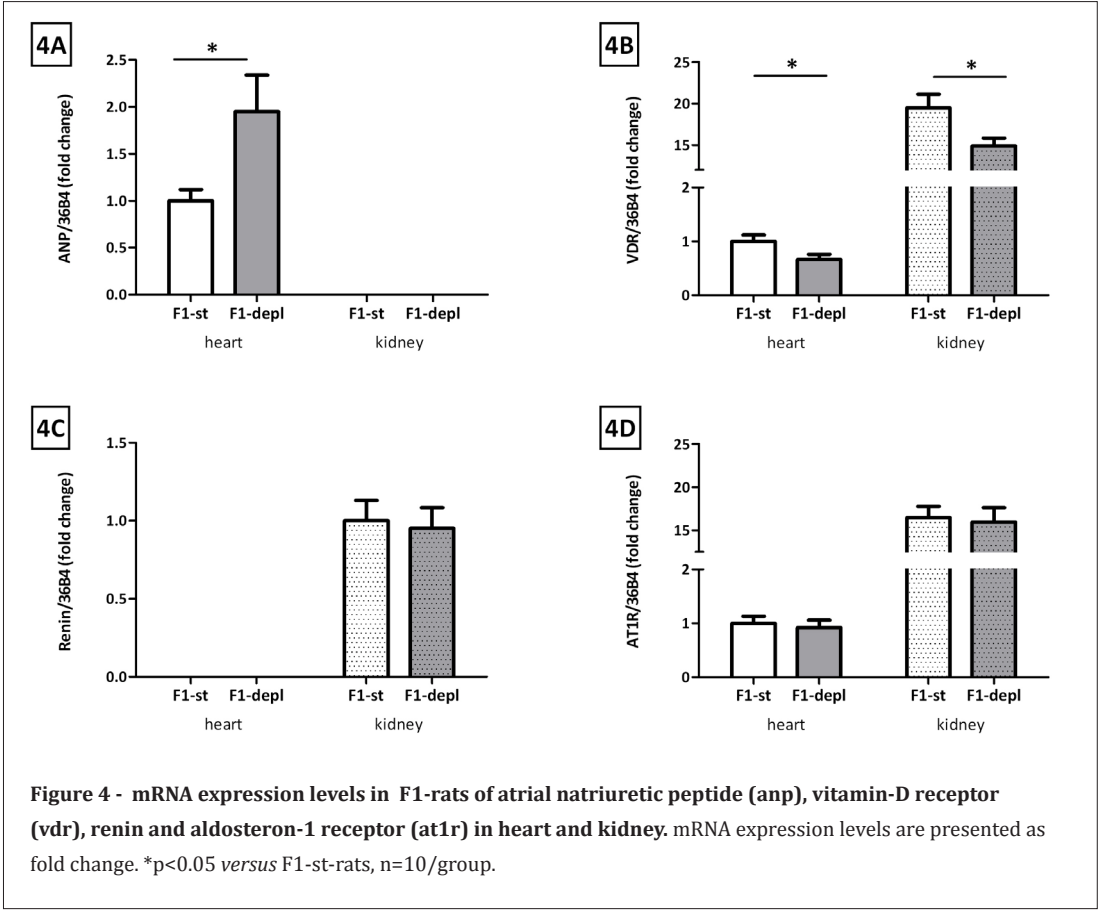
**Figure 2** - The effect of the vitamin-D depleted diet on 25-hydroxyvitamin D (25(OH)D) levels (Fig. 2A) and parathyroid hormone (PTH) levels in female F0-animals (Fig. 2B). \*p<0.05 vs. F0-st, \*\*p<0.01 vs. F0-st rats, n=3/group.

*F1-vitD-depl rats have elevated systolic and diastolic blood pressure and impaired endothelial relaxation.* Vitamin D deficient diet had no effect on blood pressure levels in the parents (Supplemental Figure 1). Single blood pressure measurement revealed that F1-depl-rats had a significant higher systolic and diastolic blood pressure than F1-st-rats (Figure 3A). Continuous blood pressure measurements for 3 days in F1-rats and confirmed elevated systolic and diastolic blood pressure in F1-depl-rats (Figure 3B).



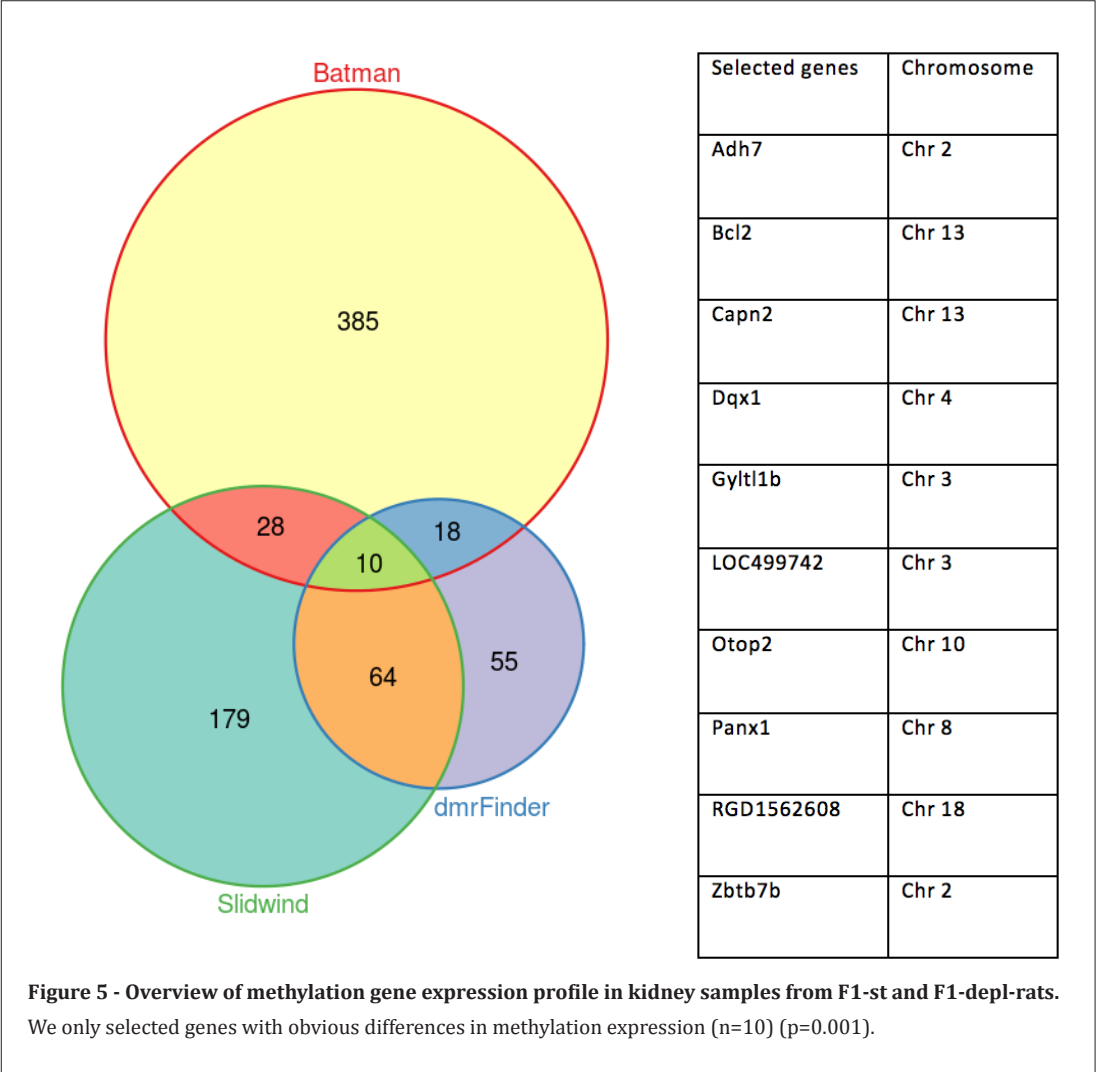
PE induced vasoconstrictor activity in isolated aorta rings was similar in all F1-rats (Figure 3C). In the aortas of F1-rats we observed a significant difference in Acetylcholine (Ach)-induced (endothelium dependent) relaxation of F1-depl-rats as compared to F1-st-rats (Figure 3C). Endothelial-independent relaxation to sodium nitroprusside (SNP) did not differ between both groups (i.e.  $3\pm2\%$  vs.  $7\pm4\%$  of pre-constriction for F1-st and F1-depl-rats respectively:  $p=ns$ ). These results suggest that the increase in blood pressure in F1-depl-rats may be a consequence of impaired endothelial relaxation.

The increase of blood pressure in F1-depl-rats is not associated with increased expression of genes of the renin-angiotensin-aldosterone system. We performed RT q-PCR to study the effect of parental vitamin D deficiency on gene expression levels of the offspring. Atrial natriuretic peptide (*Anp*) a marker of cardiac stress was increased in F1-depl-rats as compared to F1-St-rats (Figure 4A). Vitamin-D receptor (*Vdr*) expression was reduced in hearts and kidneys of F1-depl-rats (Figure 4B). We observed no changes in mRNA expression levels of renin and angiotensin-1-receptor (*at1r*). These results indicate that the increase in blood pressure is not associated with increased expression of genes of the renin-angiotensin-aldosterone system (RAAS) (Figure 4 C-D).



Parental vitamin D depletion induces epigenetic changes in the offspring. We determined DNA-methylation status (Nimblegene Chip Array) in kidney samples from offspring (3 male animals per dietary arm). Although the F1-rats were genetically identical and all fed and raised under the same conditions, we observed changes in the methylation status. As

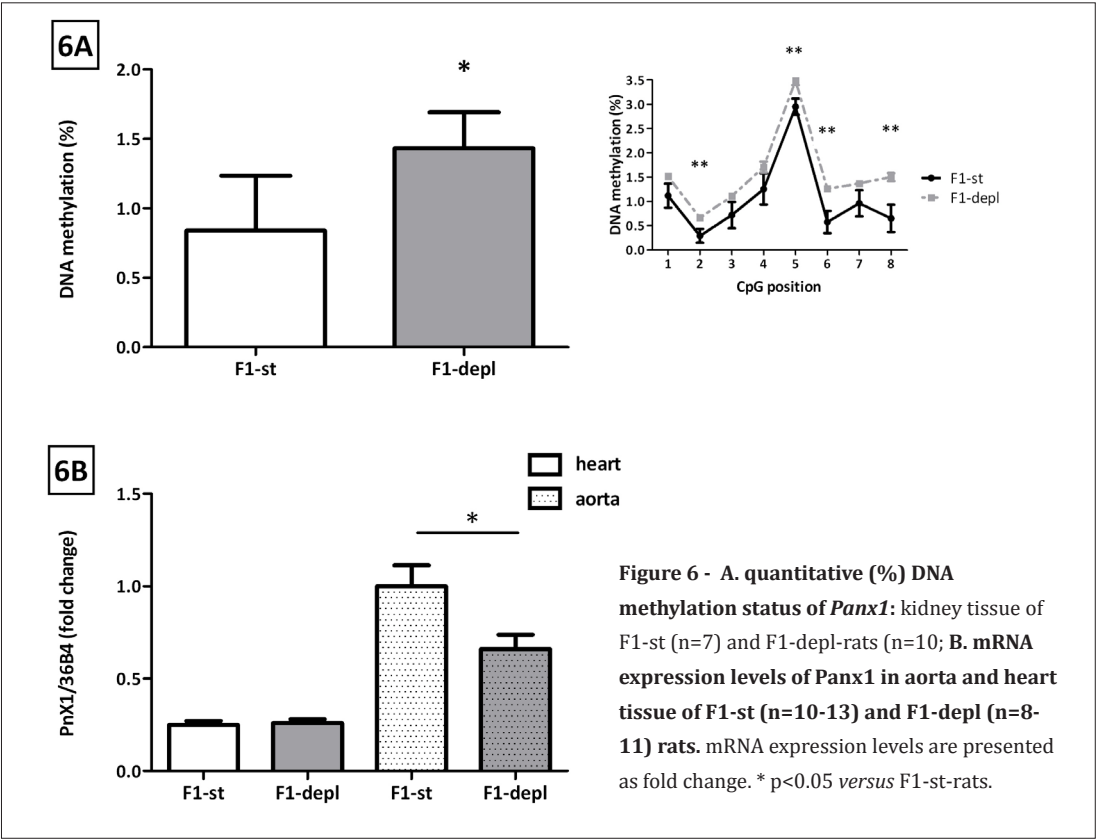
illustrated in Figure 5, we only selected those genes with an obvious change in methylation levels (n=10,  $p \leq 0.001$ ) (Figure 5).



Subsequently, we checked NCBI Gene database to obtain more information on these 10 genes. First, we found that all genes were conserved in humans. For 2 genes, RefSeq status was predicted and the rest was provisional. Functional description of 6 genes (Dqx1, Gylt1b, LOC499742, Otop2, RGD1562608, Zbtb7b) was not provided. The Capn2 and Bcl2 gene were associated with regulation of apoptosis, while the functional description of Adh7 gene was within alcohol dehydrogenase. Finally, the Pannexin-1 gene (*Panx1*) encodes for the plasma membrane protein called Pannexin-1, which is involved in various

physiological and pathophysiological processes (24). The hemichannel Pannexin-1 is abundantly present in different organs and tissues in mammals, including the heart, skeletal muscle and vasculature. Within the vasculature, Pannexin-1 is predominantly expressed in the endothelium of the larger vasculature and is associated with regulation of endothelial function (25). Therefore, we nominated *Panx1* as the most plausible candidate gene to take forward for further analyses of epigenetic changes.

*Role of Pannexin-1 in elevated blood pressure levels.* To verify if altered DNA methylation status of *Panx1* could potentially be associated with the observed endothelial relaxation in F1-depl-rats, we used pyrosequencing to perform a quantitative DNA methylation analysis on methylation status of *Panx1*. In this analysis we validated the microarray data using an extended set of samples (tissue of 10 kidneys/group) that included additional tissue from sibling and nonsibling rats. We observed a significant hypermethylation of the *Panx1* in F1-depl-rats (vs. F1-st-rats, Figure 6A). In general, hypermethylation in the promoter region causes gene silencing of genes and this we could confirm in the aortas of our rats: mRNA expression levels of *Panx1* were lower in F1-depl-rats *versus* F1-st-rats ,  $p<0.05$ , Figure 6B.



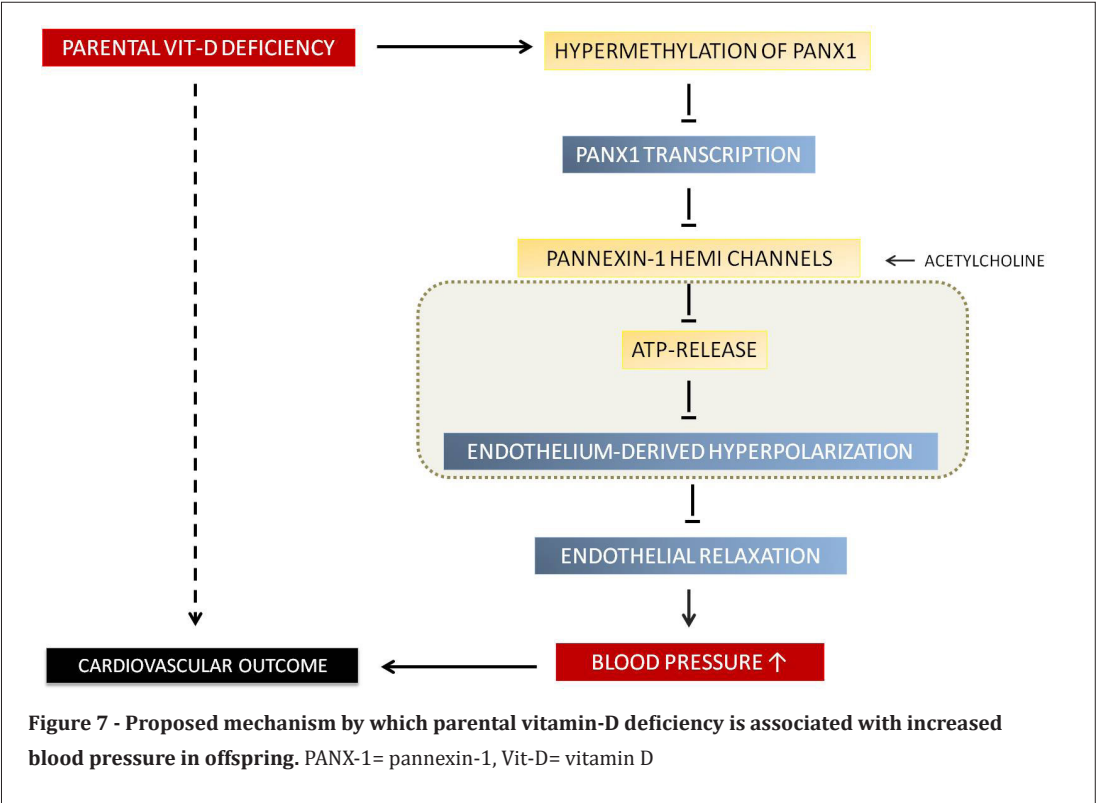
## Discussion

In this study, we show for the first time that a rat model of parental vitamin D deficiency is associated with elevated blood pressure levels in the offspring, which is accompanied by impaired endothelial relaxation in the large vessels. We propose that hypermethylation of the *Panx1* promoter-region, resulting in lower gene expression, is an underlying potential mechanism. The observed upregulation of *Anp* in heart suggests increased cardiac stress, while the reduced expression levels of the *Vdr* in heart and kidney suggests reduced VDR-activity. Our findings add to the mounting literature suggesting that nutritional deficiencies of parents may translate into enhance susceptibility to several common multifactorial diseases in their children.

A recent large multi-ethnic genome-wide association and replication study provided new insights regarding genetic predisposition of blood pressure regulation, and suggested DNA methylation to be an important player in this process (26). Previously, it has been shown that low levels of vitamin are associated with increased risk of hypertension (27-29), and this has been confirmed recently (30). This suggests a direct relationship between vitamin D biology and blood pressure regulation. Interestingly, maternal vitamin D deficiency has been associated with changes in methylation status of the offspring as well (31). Therefore, it is conceivable that vitamin D may intervene with blood pressure levels in an indirect manner as well.

DNA methylation is part of the process of epigenetic modifications that control how genes are expressed in an individual without altering the DNA. Especially during gametogenesis and early embryogenesis this process has a major impact on interindividual variation in gene expression in various organs and tissues. This is thought to contribute to altered susceptibility to certain diseases later on in life (32). Epigenetic modifications can be provoked by numerous factors, including environmental chemicals, drugs, but also nutrition (33,34). Nutritional factors, in particular maternal deficiencies, are therefore regarded as well-known modifiers of long-term disease outcomes in offspring. It has repetitively been reported that extreme maternal food deprivation during the first trimester of pregnancy induced increased cardiovascular disease prevalence, risk of metabolic disorders and breast cancer in their children (35-37). Interestingly, maternal nutritional deficiencies are also directly associated with blood pressure regulation in offspring: the offspring from rats and mice fed on a low protein diet tend to develop increased blood pressure levels (38,39), and it has been shown that children from malnourished mothers had an increased susceptibility to develop hypertension later in their lives (36). However, no clear cause- and effect relationship explaining the development of hypertension has been established yet (40). In our study, parental vitamin D deficiency is associated with increased blood pressure in

F1 animals, which was not in parallel and thus independent of the parental blood pressure. Since the increase in blood pressure, in this study, was not associated with changes in expression of genes of the RAAS, we hypothesize that this would be due to another *in-utero* programming effect, such as altered DNA-methylation status. We assessed DNA methylation status using quantitative and qualitative methods and identified Panx1 hypermethylation of the promoter region and reduced gene expression in the aorta but not in the heart.



The pannexin (Panx) family exists out of three isoforms (Panx1, Panx2, and Panx3) that are present throughout the entire body including the endothelial cells of the large vasculature. In the vessel wall, Panx1 is most abundantly expressed (41). *Panx1* is thought to form ATP-permeable hexameric channels that release ATP. Binding of Ach to its receptor on the endothelial surface activates Panx1-hemichannels that induce endothelium-derived hyperpolarization (EDH) (24,41). Interestingly, mice that lack the *Panx1* encounter endothelial dysfunction due to impaired Ach-induced relaxation (25), and functional Panx1-hemichannels seem to be essential in EDH-like relaxation (42). We hypothesize that the reduction in *Panx1* expression may explain, at least in part, the observed differences in blood pressure levels in F1-rats. From our aggregate data, we suggest that parental



vitamin-D deficiency causes hypermethylation in the promoter region of *Panx1*. This results in less *Panx1* transcription and gene expression, and inhibition of EDH. As a consequence, Ach-induced relaxation is impaired: relaxation of the large vessels is hampered and blood pressure levels increase (Figure 7).

Vitamin D levels and VDR are thought to play an essential role in endothelial function as well. Previously, it has been demonstrated that VDR activation of uremic rats significantly improved endothelial function without alteration of blood pressure (43-46). Given the observation that we observed an increased blood pressure in F1-depl-rats, we suggest that the effect of reduced *Vdr* is unlikely a major factor in explaining our results.

This study has several limitations: (i) global methylation status is measured on kidney tissue and not on vascular segments or endothelial cells; (ii) because of the descriptive nature of this study, we are unable to prove a direct cause and effect relation of parental vitamin D deficiency and the development of increased blood pressure in the offspring; (iii) in this study we show that the increase in blood pressure levels is associated with a decrease of mRNA expression levels of *Panx1* in the thoracic aorta. Besides larger arteries, smaller resistance vessels (arterioles) also regulate blood pressure levels. *Panx1* is present in both arteries and arterioles, and it has been hypothesized that *Panx1*-activity regulates vascular tone, peripheral resistance, and blood pressure levels in larger and smaller vessels (47). Future studies should address if altered *Panx1*-activity in smaller resistance vessels influence blood pressure levels as well.

In conclusion, we demonstrate that parental vitamin D deficiency is associated with increased blood pressure in offspring. As a potential and plausible biological mechanism, we suggest *Panx1* hypermethylation and a concomitant reduction in gene expression to be contributing to the observed disturbed endothelial relaxation. This phenomenon may not only directly result in higher blood pressure, but may eventually also contribute to adverse cardiovascular outcomes later in life.

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The authors have nothing to declare.

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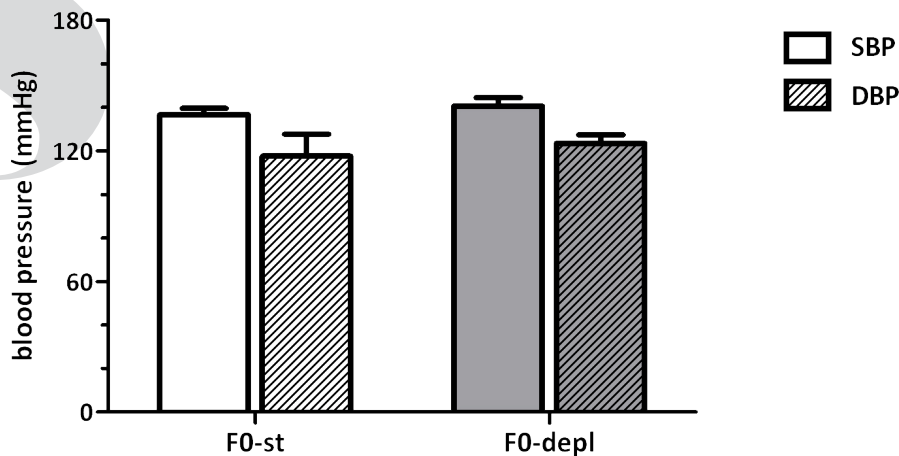
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# Supplemental data and figures



Supplemental Figure 1 - Blood pressure levels in male and female F0-rats (n=6 per group).

